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Disruption of T Cell Tolerance to Self-Immunoglobulin Causes Polyclonal B Cell Stimulation Followed by Inactivation of Responding Autoreactive T Cells[1]

Arpita Choudhury, Paushali Mukherjee, Sandip K. Basu, Anna George, Satyajit Rath,[2] and Vineeta Bal[2]

Scavenger receptor (SR)-specific delivery by maleylation of a ubiquitous self-protein, Ig, to SR-bearing APCs results in self-limiting induction of autoimmune effects in vivo. Immunization with maleyl-Ig breaks T cell tolerance to self-Ig and causes hypergammaglobulinemia, with increases in spleen weight and cellularity. The majority of splenic B cells show an activated phenotype upon maleyl-Ig immunization, leading to large-scale conversion to a CD138$^+$ phenotype and to significant increases in CD138-expressing splenic plasma cells. The polyclonal B cell activation, hypergammaglobulinemia, and autoreactive Ig-specific T cell responses decline over a 2-mo period postimmunization. Following adoptive transfer, T cells from maleyl-Ig-immune mice taken at 2 wk postimmunization can induce hypergammaglobulinemia in the recipients, but those taken at 10 wk postimmunization cannot. Hypergammaglobulinemia in the adoptive transfer recipients is also transient and is followed by an inability to respond to fresh maleyl-Ig immunization, suggesting that the autoreactive Ig-specific T cells are inactivated peripherally following disruption of tolerance. Thus, although autoreactive T cell responses to a ubiquitous self-Ag, Ig, are induced by SR-mediated delivery to professional APCs in vivo resulting in autoimmune pathophysiological effects, they are effectively and rapidly turned off by inactivation of these activated Ig-specific T cells in vivo. *The Journal of Immunology,* **2000, 164: 1713–1721.**

The pathogenesis of diseases considered autoimmune depends crucially on the generation of a self-reactive immune response despite the presence of mechanisms maintaining a state of immune self-tolerance. Normally, both the B and T cells are negatively selected during their development, and autoreactive cells can be eliminated by apoptotic deletion (1–4). Deletional mechanisms operate more stringently for T cells rather than for B cells (5, 6), and hence the frequency of finding autoreactive B cells in the mature peripheral repertoire is higher than that for the T cells (6–9). In the periphery, autoreactive mature B and T cells that have escaped deletion are turned off by a variety of mechanisms (10–13). Despite multiple mechanisms being available for silencing autoantigen-specific immunocytes, their presence can be documented in normal individuals (14, 15), suggesting leakiness in the mechanisms inducing and maintaining self-tolerance. Such autoreactive B and T cell responses can be detected with increasing frequency in aging populations (16–18), even in the absence of any demonstrable pathological consequences (15, 18). Thus, autoreactive immunocytes by themselves, even when triggered in vivo, may not be sufficient for inducing autoimmune disease.

Naive T cells are activated when professional APCs present Ag and deliver Ag-nonspecific costimulatory signals (19–21). The density and duration of availability of peptide-MHC complexes, which constitute the first signal for T cell activation, have been suggested to be important for optimal T cell activation (22, 23). The presence or absence of costimulatory molecules of the B7 family (CD80, CD86) on the priming APCs has also been shown to modify the outcome of TCR-mediated T cell activation (24–26). It has been documented that a large proportion of peptides normally present on MHC molecules are derived from APC endogenous sources (27–29). There have been suggestions that, for those T cells that have been anergized, the persistent presence of their target peptide-MHC complexes on peripheral professional APCs is necessary to maintain a state of anergy (30).

In this context, we have been trying to examine how autoimmune T cell responses against a ubiquitous self-Ag induced in vivo by disrupting normal T cell tolerance are regulated. For this purpose, we have used maleylated self-Ags. Protein maleylation confers on them the ability to bind to scavenger receptors (SRs)[3] (31–33). Although classical SRs are restricted to cells of the monocytic lineage such as macrophages and dendritic cells (DCs) (33, 34), other receptor molecules with the ability to bind SR ligands have also been shown to be present on B cells (34, 35). Thus, protein maleylation allows the specific delivery and presentation of the protein to all APC lineages, B cells, macrophages, and DCs (35), inducing enhanced T cell immunity (36). Using the maleylated self-Ag mouse serum albumin (MSA) for immunization, we have shown earlier that an effective T cell response recognizing multiple MSA-derived peptides can be demonstrated in vitro (37). However, despite the presence of these activated MSA-specific T cells as well as of MSA-peptide-loaded APCs, no autoimmune phenomena could be demonstrated in vivo. It was therefore also of interest

National Institute of Immunology, New Delhi, India

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[2] Address correspondence and reprint requests to Dr. Satyajit Rath or Dr. Vineeta Bal, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110 067, India. E-mail addresses: satyajit@nii.res.in or vineeta@nii.res.in

[3] Abbreviations used in this paper: SR, scavenger receptor; CA, conalbumin; CGN, carrageenan; cyt-*c*, cytochrome *c*; DC, dendritic cells; MSA, mouse serum albumin; PNA, peanut agglutinin.

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to examine whether this was because the self-reactive T cells triggered by APC-specific delivery of maleylated self-Ags were incapable of functioning in vivo, or because Ag-specific factors determine the in vivo reactivity and the pathophysiological consequences of the autoreactive T cells triggered.

In this study, we report use of another self-protein, Ig, which, unlike MSA, is normally synthesized by one class of MHC class II-expressing APCs, the B cells. This was also of interest in the context of the reported data that though naive B cells can induce T cell anergy (38), activated B cells can break T cell tolerance to a self-Ag they are specific for (39). Our results using maleyl-Ig as an immunogen show that self-Ig-specific T cells can be generated in vivo, that the break in T cell tolerance against Ig causes short-lived polyclonal B cell activation and hypergammaglobulinemia in vivo, and that the activated Ig-specific T cells are inactivated following their triggering.

Materials and Methods

Mice, immunization, and cell transfers

BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), bred in the small animal facility of the National Institute of Immunology (New Delhi, India) were used for the experiments. Five to seven mice per group were immunized i.p. with a single dose of 300 μg of native or maleylated Ag in PBS or adsorbed on alum (Alhydrogel; Superfos, Vedback, Denmark). For certain experiments, 300 μg of maleyl-poly-L-lysine-Ig (see method of preparation below) was mixed with 1 mg of poly(G) or poly(C) (Sigma, St. Louis, MO) before immunization. Mice were immunized with conalbumin (CA) or maleylated cytochrome *c* (cyt-*c*; Sigma) on alum. 300 μg mouse i.p., as indicated. Mice were bled from the retro-orbital plexus under inhalation anesthesia, and sera were used for the estimation of Ab levels. Mice were killed by cervical dislocation and single-cell suspensions from spleens were used for flow cytometric analysis, cell fractionation, and T cell proliferation assays. Where appropriate, various cell combinations were injected i.p. in syngeneic recipients. The recipients were γ-irradiated (300 rad) BALB/c mice. Wherever appropriate, the recipient mice were immunized i.p. with maleyl-Ig in PBS (300 μg/mouse). All animal experimentation was done using protocols approved by the Institutional Animal Ethics Committee.

Chemical modification and conjugation of proteins

Pooled sera of normal BALB/c mice were salt precipitated with 40% ammonium sulfate, and the precipitate was dissolved in PBS, dialyzed, and applied to a protein A column (Pharmacia Biotech, Uppsala, Sweden) for further purification. The protein thus obtained was analyzed on a 10% reducing SDS-polyacrylamide gel with appropriate m.w. markers to ascertain purity. Protein concentration was estimated with bicinchoninic acid (Sigma). MSA (Sigma), poly-L-lysine (Sigma), Ig, or cyt-*c* were maleylated with maleic anhydride (Sigma) at alkaline pH as described previously (40), followed by dialysis against PBS. The degree of maleylation was estimated from the loss of free ϵ-amino groups measured using trinitrobenzenesulfonic acid (41). Only protein preparations with >90% maleylation were used for the experiments.

Poly-L-lysine or its maleylated form were mixed in a ratio of 3:1 with Ig and chemical coupling was conducted by adding aqueous glutaraldehyde (Sigma) dropwise to a final concentration of 0.1%. The conjugates were used for immunization after extensive dialysis.

T cell proliferation assays

Spleen cells from immune mice were cultured with graded doses of Ag in 96-well flat-bottom microtiter plates (Corning Glass, Corning, NY) at 3–5 $\times$ 10^5 cells/well in 200 μl of L-glutamine-fortified Click's medium (Irvine Scientific, Irvine, CA) containing 5 $\times$ 10^{-5} M 2-ME (Life Technologies, Grand Island, NY), antibiotics and 10% FCS (Life Technologies). The wells were pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) for 12–14 h in a 96-h assay and harvested onto fiberglass filter mats for scintillation counting (Betaplate; Wallac, Turku, Finland). Results are expressed as cpm (mean $\pm$ SE) of triplicate cultures.

Triplicate cultures of thymoctes (5 $\times$ 10^5 cells/well) were stimulated by a submaximal concentration of an anti-CD3ϵ mAb (500-A2; a gift from Dr. C. A. Janeway, Yale Medical School, New Haven, CT) in the presence of splenic DC populations isolated from mice treated with carrageenan as described below or from control mice. The wells were pulsed after 48 h with [^{3}H]thymidine, and proliferative responses were measured and expressed as above.

Carrageenan treatment for macrophage depletion

Macrophage depletion in vivo was done by injecting *i*-carrageenan (CGN; Sigma) (1 mg/mouse) i.p. every second day (42). On the eighth day, mice were immunized with Ig or maleyl-Ig as described. The protocol was optimized by varying dosage and schedule to ensure complete depletion of macrophages as judged by absence of Mac-1-bearing cells using anti-CD11b mAb (TIB128; American Type Culture Collection (ATCC)) in the spleen on the day of immunization and for 5 succeeding days (data not shown). This in vivo treatment did not deplete B cells and DCs as seen by flow cytometry. DCs were also isolated from splenic cells of carrageenan-treated or control mice on the eighth day as described below and used as APCs for anti-CD3-induced thymocyte proliferation.

Ab assays

Estimation of total Ig in the sera was done by enzyme immunoassays. Polyvinyl chloride microtiter plates (Falcon; Becton Dickinson, Lincoln Park, NJ) were coated with affinity-purified goat anti-mouse Ig (10 μg/ml) and test sera were titrated. The bound serum Ig was detected by adding goat anti-mouse Ig peroxidase, followed by *o*-phenylenediamine (Sigma) and hydrogen peroxide as revealing agents. Absorbance was read at 492 nm in a microplate reader (Bio-Tek, Vinooski, VT). For determination of IgM, goat anti-mouse-IgM peroxidase was used for detection. For estimation of total IgG, biotinylated anti-mouse-IgG followed by avidin-peroxidase constituted the detection system. For estimation of CA-specific Abs, plates were coated with CA (10 μg/ml), test sera were titrated, and bound Ig was detected using goat anti-mouse Ig peroxidase. Concentrations were calculated, in each instance, on the basis of a known standard titrated in parallel to the serum samples.

Separation of splenic cell subpopulations

Pooled splenic cells from appropriate groups of mice were incubated on plastic plates (Corning Glass) for 1 h at 37°C to remove macrophages. The nonadherent population was separated into B and T cells using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, the nonadherent population was stained with anti-B220-biotin on ice for 45 min, washed, and streptavidin-labeled magnetic beads (Miltenyi Biotec) were added. After a 15-min incubation on ice, the cell suspension was applied to the magnetic column and nonadherent T cells were washed through into the effluent. The column was then removed from the magnetic field and adherent B cells were flushed out. The purity of the separated B and T cell populations was checked by flow cytometry with anti-B220 for B cells and anti-Thy-1 for T cells, and was commonly >95%. The separated cell populations were washed several times with Click's medium containing 10% FCS. Alternatively, plastic nonadherent splenic cells were loaded onto a nylon wool column and nylon wool nonadherent cells were collected as T cells, checked for purity using flow cytometry, and used for additional assays. Purified T cells from Ig-immune or maleyl-Ig-immune mice and B cells separated from naive syngeneic mice were mixed in a 3:1 ratio and injected i.p. into recipient mice where appropriate.

DCs were isolated from carrageenan-treated or control mice using negative selection by removing cells stained with mAbs against Thy-1, B220, and Mac-1 followed with goat anti-rat Ig-coated magnetic beads on magnetic columns (Miltenyi Biotec). The mAb specific for the B cell-specific isoform of CD45 (B220), 6B2, the anti-Thy-1 mAb Y-19, and the anti-Mac-1 mAb M17/4.2 (kind gifts from Dr. C. A. Janeway, Yale Medical School) were used as culture supernatants.

Flow cytometry

For flow cytometry, cells (1 $\times$ 10^5–1 $\times$ 10^6/well) were incubated with the primary staining reagents in 100 μl for 45 min on ice. Heat-aggregated serum of the appropriate species (rat or goat) was used at 1% in the staining buffer (PBS containing 0.1% sodium azide and 1% BSA) to block Fc receptors. After washing, a similar procedure was followed for the secondary and tertiary reagents wherever necessary. The mAb specific for the B cell-specific isoform of CD45 (B220), 6B2, and the anti-Thy-1 mAb Y-19 were used as either culture supernatants or in the biotinylated form. IgD expression and peanut agglutinin (PNA) binding were detected by using fluorescein-coupled anti-IgD or PNA (Accurate, San Diego, CA). CD138 was detected using an anti-CD138 mAb (PharMingen, San Diego, CA). Splenic DCs were detected using the hamster mAb N418 (HB224; ATCC) and macrophages by the rat mAb specific for Mac-1, M17/4.2 (TIB217; ATCC). Primary unlabeled mAbs of rat or hamster origin were detected

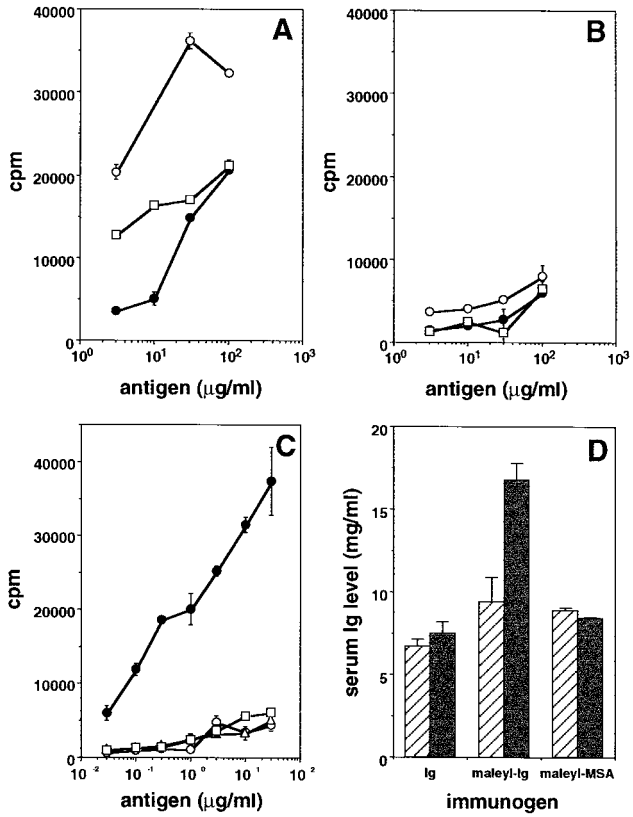

FIGURE 1. Immunization with maleyl-Ig results in autoreactive anti-Ig T cell responses and hypergammaglobulinemia. Proliferative responses of splenic cells from maleyl-Ig-immune (*A*) and Ig-immune (*B*) mice to maleyl-Ig (○), Ig (□), and autologous preimmune serum (●) are shown. The background counts were <3500 cpm. The results are representative of seven experiments. *C*, Proliferative responses of splenocytes from maleyl-cyt-*c*-immune mice to maleyl-cyt-*c* (●), maleyl-Ig (○), maleyl-MSA (△), and maleyl-OVA (□). The results are representative of four experiments. *D*, Serum Ig levels from Ig-immune, maleyl-Ig-immune, and maleyl-MSA-immune mice on day 0 (▨) and day 14 (■) postimmunization (mean ± SE). The data represent three independent experiments.

using goat anti-rat or anti-hamster IgG-FITC, respectively, whereas biotinylated reagents were detected using streptavidin-PE (Jackson ImmunoResearch, West Grove, PA). Stained cells were either analyzed immediately or fixed with 0.1% paraformaldehyde (Sigma) and stored at 4°C until analyzed on a flow cytometer (Bryte; Bio-Rad, Hemel Hampstead, U.K.). Data analysis was done with FlowJo software (Treestar, San Jose, CA), or with WinMDI shareware.

Statistical analysis

Wherever appropriate, statistical analysis was conducted using Student's *t* test.

Results

Immunization with maleyl-Ig breaks T cell tolerance to Ig and causes hypergammaglobulinemia in vivo

We have shown earlier that immunization with maleyl-MSA results in a break in T cell tolerance of MSA (37). Whether this was an exclusive finding for MSA or held true for other ubiquitous self-proteins was tested using Ig in its native or maleylated form for immunization. Seven days after immunization with 300 μg of maleyl-Ig or Ig on alum given i.p., mice were sacrificed. Fig. 1 shows proliferation of splenic cells from mice immunized with either maleyl-Ig (*A*) or native Ig (*B*) in response to titrated Ag doses. Splenic cells from maleyl-Ig-immune mice responded well not only to maleyl-Ig but also to Ig, albeit to a lesser extent. Interestingly, these cells from individual maleyl-Ig-immune mice also responded to preimmune serum from the same mouse. In contrast, cells from Ig-immune mice did not respond significantly to any of the three Ags used for recall.

To confirm that the proliferative response induced by maleyl-Ig was Ag specific and not due to some nonspecific mitogenic effect of maleyl-Ig, T cells from mice immunized with an unrelated maleylated self-protein, maleyl-cyt-*c*, were stimulated with various maleylated Ags, self- as well as nonself. Maleyl-Ig did not evoke any response from maleyl-cyt-*c*-immune cells despite an excellent response to maleyl-cyt-*c* itself (Fig. 1*C*), confirming, as we have shown earlier (36), that maleyl proteins do not have nonspecific T cell mitogenic activity. Thus, immunization with maleyl-Ig results in a break in T cell tolerance for self-Ig.

Since Ig-specific T cells can potentially help Ig-expressing B cells and induce Ig secretion, the levels of total serum Ig in the maleyl-Ig-immune and Ig-immune groups were examined. Although there was no change in serum Ig levels in Ig-immune mice at 2 wk postimmunization, the serum Ig levels of maleyl-Ig-immune mice showed a significant ($p < 0.05$) increase (Fig. 1*D*). However, immunization with maleyl-MSA, which results in a break in T cell tolerance to MSA (37), did not lead to any increases in serum Ig levels (Fig. 1*D*), supporting the notion that the effect observed is an Ag-specific B cell differentiation to plasma cells and not a nonspecific inflammatory component of an autoimmune response.

Targeting of Ags to scavenger receptors in vivo is crucial for a break in tolerance

In addition to maleylated proteins, SRs bind to many other ligands (31–33, 40), and we have shown previously that SR-like binding capabilities are exhibited by all professional APCs (35), although classical SRs are known to be present only on cells of the monocytic lineage (31, 33, 34). To examine the role of macrophages in presentation of self-Ags to generate autoimmune responses, we used CGN to deplete macrophages of their functional capability at the time of immunization. A week after immunization with maleyl-Ig, mice were sacrificed and T cell responses to maleyl-Ig were analyzed. Fig. 2*A* shows the responses of splenic cells from two normal mice and two CGN-treated mice immunized with maleyl-Ig 7 days earlier. Although normal mice showed significant responses to maleyl-Ig, CGN-treated mice mounted very weak responses to maleyl-Ig immunization. Unlike macrophages, DCs are not depleted by carrageenan treatment, whereas macrophages are efficiently depleted (Fig. 2*B*). We also tested the DCs from carrageenan-treated mice to exclude the possibility that they were functionally compromised by carrageenan. DC-enriched cells from carrageenan-treated or control mice were equally efficient in supporting anti-CD3-mediated stimulation of thymocytes (Fig. 2*C*). This supported the possibility that the partial loss of response to maleyl-Ig in carrageenan-treated mice was likely to be due to the absence of macrophages, suggesting that macrophage APCs may play a significant role in breaking T cell tolerance via maleylated self-Ags.

As an alternative approach to maleylation of the immunogen, unmodified Ig was coupled to either maleylated or unmodified poly-L-lysine and used as the immunogen (300 μg/mouse in PBS). Fig. 2*D* shows that Ig coupled to unmodified poly-L-lysine does not alter the serum Ig levels in immunized mice. However, like maleyl-Ig immunization, immunization with Ig coupled to maleyl-poly-L-lysine induces substantial hypergammaglobulinemia on day 14 postimmunization. Addition of a 3-fold excess of the SR ligand, poly(G) (40), to the maleyl-poly-L-lysine-Ig during immunization results in marked inhibition of hypergammaglobulinemia ($p <$

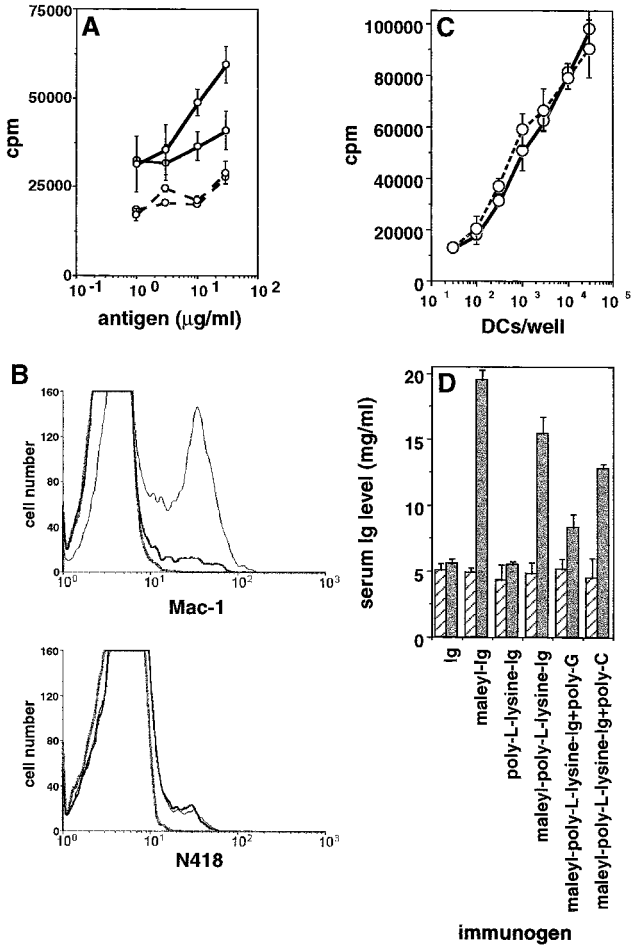

FIGURE 2. Break in tolerance to Ig is dependent on targeting of Ig to SR-bearing APCs such as macrophages. *A*, Responses of individual maleyl-Ig-immune mice to maleyl-Ig when mice were pretreated (broken line) or not pretreated (continuous lines) with CGN. Background counts were 7000–8000 cpm. The results are representative of two independent experiments. *B*, Flow cytometric analysis of spleen cells from mice pretreated (thick lines) or not pretreated (thin lines) with CGN, stained for either Mac-1 or the DC marker N418 as indicated. Negative control curves are shown in gray. The results are representative of five separate experiments. *C*, Response of thymocytes to anti-CD3ϵ in the presence of titrating numbers of DCs from mice pretreated (broken line) or not pretreated (continuous line) with CGN. Background counts were 3000–5000 cpm. The data are representative of two experiments. *D*, Serum Ig levels (mean ± SE, for five mice per group) on day 0 (▨) and day 14 (■) postimmunization from variously immunized mice as shown. Data represent three independent experiments.

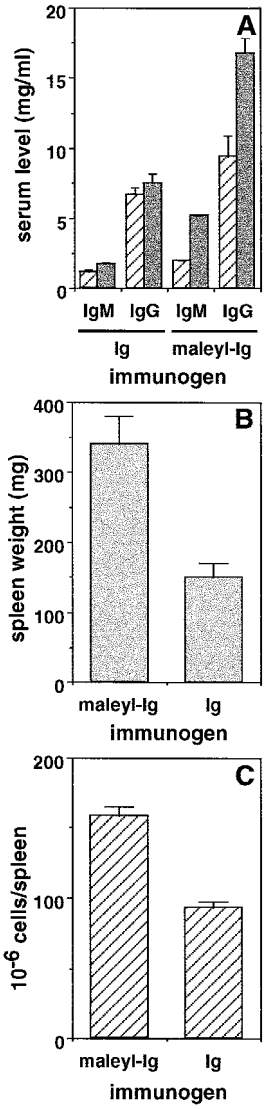

FIGURE 3. Break in tolerance to Ig results in B cell activation in vivo. *A*, IgM and IgG levels (mean ± SE) on day 0 (▨) and day 14 (■) postimmunization from Ig-immune and maleyl-Ig-immune mice. *B*, Average spleen weight (mean ± SE). *C*, Spleen cell counts (mean ± SE) from various groups on day 7 postimmunization. The data represent four independent experiments.

0.05), whereas addition of a related molecule, poly(C), which is not an SR ligand (40), does not do so ($p > 0.4$), suggesting that delivery of maleyl-poly-L-lysine-Ig via SRs is likely to be critical for the effects seen in vivo.

Maleyl-Ig immunization leads to polyclonal B cell activation in vivo

The Ig isotypes affected by hypergammaglobulinemia were next examined, and it can be seen that both IgM and IgG levels went up at 2 wk postimmunization in maleyl-Ig-immune mice (Fig. 3*A*) as compared with those seen in Ig-immune mice ($p < 0.01$). Since both primary and secondary Ig isotypes are affected, the B cell stimulation observed is likely to be T cell dependent. In addition to the hypergammaglobulinemia, on day 7 postimmunization, spleens from maleyl-Ig-immune mice showed an increase in weight ($p < 0.01$) as well as cellularity ($p < 0.05$) as seen in Fig. 3.

If the serum hypergammaglobulinemia seen in maleyl-Ig-immune mice is the result of activated Ig-specific T cells triggering Ig-presenting B cells in vivo, practically all B cells in these mice would be expected to be affected. To test this directly, we next examined the phenotype of splenic B cells by two-color flow cytometry. Splenic cells from maleyl-Ig-immune or Ig-immune mice were stained 1 wk postimmunization for the B cell marker B220 vs IgD or PNA reactivity as markers of B cell activation. Fig. 4*A* shows that there was a significant reduction in the proportion of IgD-bearing B cells in maleyl-Ig-immune mice (*b*) in comparison to their Ig-immunized counterparts (*a*), although the frequency of total splenic B cells was not significantly altered. Since IgD is normally down-regulated by naive B cells upon T-dependent germinal center activation (43), this suggested that most naive B cells in the spleens of maleyl-Ig-immune mice were receiving activation signals.

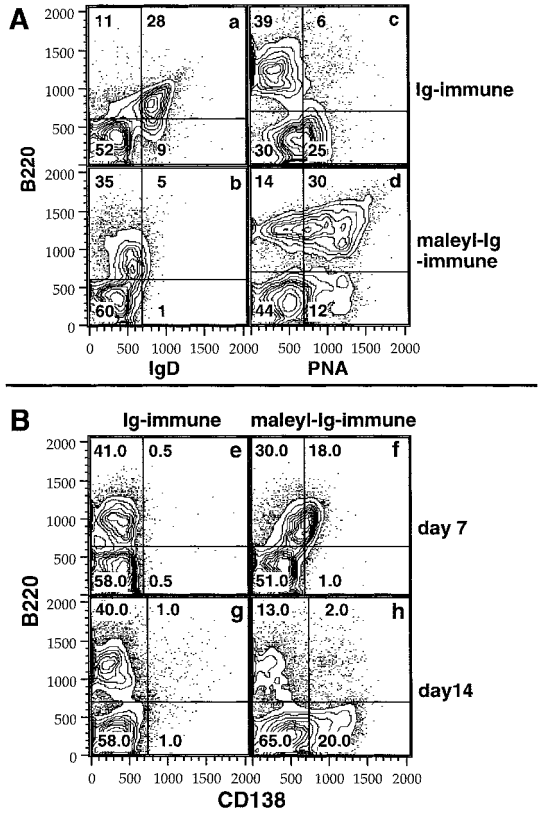

FIGURE 4. Most splenic B cells in maleyl-Ig-immune mice show an activated phenotype. *A*, Two-color flow cytometric analysis for B220 vs either IgD (*plots a* and *b*) or PNA (*plots c* and *d*) on day 7 postimmunization. *Plots a* and *c*, Staining from Ig-immune and *plots b* and *d* from maleyl-Ig-immune mice. *B*, A similar analysis for B220 vs CD138 on day 7 (*plots e* and *f*) and day 14 (*plots g* and *h*) postimmunization on splenic cells from Ig-immune (*plots e* and *g*) and maleyl-Ig-immune (*plots f* and *h*) mice. Numbers in various compartments show percentages of cells. Results represent four independent experiments.

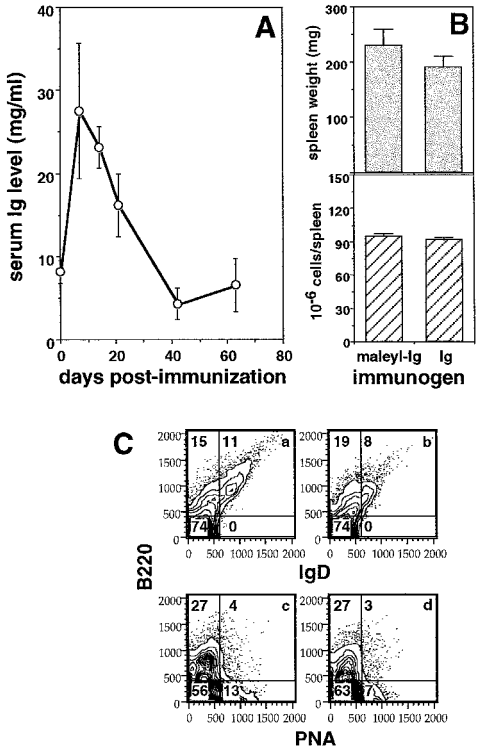

FIGURE 5. Effects of break in Ig-specific T cell tolerance are transient in nature. *A*, Serum Ig levels (mean ± SE) in maleyl-Ig-immune mice over a period of 9 wk. *B*, Spleen weight and cellularity in Ig-immune and maleyl-Ig-immune mice on day 70 postimmunization. *C*, The phenotype of splenic B cells from Ig-immune and maleyl-Ig-immune mice on day 70 postimmunization. Two-color staining patterns are shown for B220 vs IgD (*a* and *b*) or B220 vs PNA (*c* and *d*) from Ig-immune (*a* and *c*) or maleyl-Ig-immune (*b* and *d*) mice. Numbers in various compartments show percentage of cells. The data are representative of three independent experiments.

An increase in the ability to bind to the lectin PNA normally accompanies T-dependent B cell activation and germinal center formation, and PNA binding is in fact treated as a hallmark of germinal center B cells (43). As seen in Fig. 4*A*, while only a minor proportion of B cells in Ig-immune mice were PNA^+, the majority of B cells in the maleyl-Ig-immune mice acquired PNA-binding capabilities (*d*), although the frequency of total splenic B cells was again unchanged. Thus, soon after immunization, before the peak of hypergammaglobulinemia is reached, the majority of splenic B cells in maleyl-Ig-immune mice are in an activated state.

Activation of B cells must lead to their differentiation into plasma cells before they can secrete large quantities of Ig (44). To investigate whether most B cells triggered by Ig-specific autoreactive T cells activated by maleyl-Ig immunization went on to give rise to plasma cells, CD138 was used as a marker for plasma cells in flow cytometric analyses (45–47). Fig. 4*B* shows a two-color analysis for B220 and CD138 in Ig-immune (*plots e* and *g*) and maleyl-Ig-immune (*plots f* and *h*) mice on day 7 and day 14 postimmunization. There were very few $CD138^+$ splenic cells in the Ig-immune mice at either time point. In sharp contrast, maleyl-Ig-immune mice had a large proportion of their $B220^+$ cells expressing CD138 on day 7, although there were no classical $CD138^+B220^-$ plasma cells seen. By day 14 postimmunization with maleyl-Ig, however, such $B220^-$ plasma cells had become a major population (20%) in the spleen, and the frequency of $B220^+$ B cells had concomitantly declined. These data suggest that polyclonal B cell activation by autoreactive T cells in maleyl-Ig-immune mice progresses to terminal plasma cell differentiation of the majority of activated B cells.

Maleyl-Ig-induced hypergammaglobulinemia and polyclonal B cell activation is short-lived in vivo

To examine the course of the putative autoimmune effects observed in vivo, maleyl-Ig-immune mice were bled sequentially and serum Ig levels were estimated. By day 42 postimmunization with maleyl-Ig, serum Ig levels had returned to normal (Fig. 5*A*). By 10 wk postimmunization, splenic weight and cellularity were again comparable between Ig-immune and maleyl-Ig-immune groups (Fig. 5*B*). The phenotypic changes in B cells observed at early time points also disappeared by this time as shown in Fig. 5*C*. The frequency of IgD-expressing $B220^+$ B cells in maleyl-Ig-immune spleen cells (Fig. 5*C*, *b*) was now only marginally lower than that in control Ig-immune spleen cells (Fig. 5*C*, *a*), and there was little difference between the PNA-binding B cell frequencies in the two groups (Fig. 5*C*, *c* and *d*). Thus, the presence of activated splenic B cells in maleyl-Ig-immune mice decreases in concordance with the reduction in the serum hypergammaglobulinemia.

Functional inactivation of Ig-specific T cells in vivo over time

We next investigated whether Ig-specific T cells that are presumably responsible for the observed hypergammaglobulinemia persist in vivo. Splenocytes from mice immunized with Ig or maleyl-Ig and sacrificed on day 70 postimmunization showed hardly

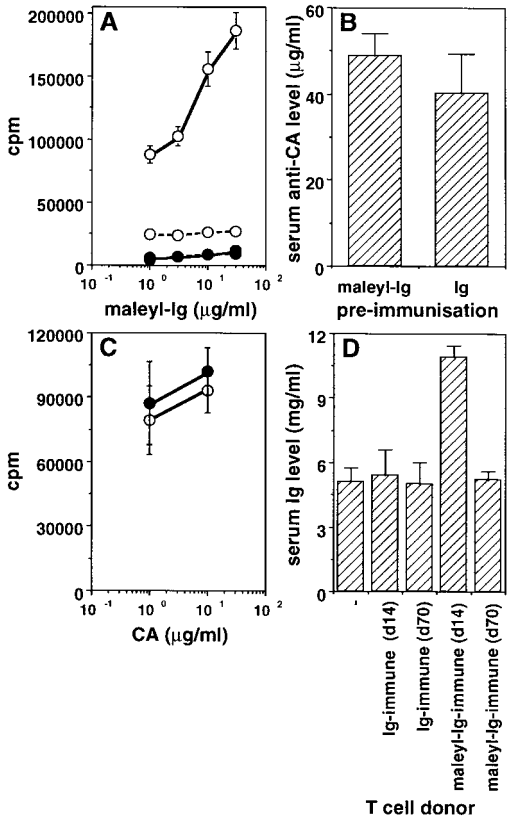

FIGURE 6. Specific loss of Ig-specific T cell function in vivo. *A*, T cell-proliferative responses in vitro from Ig-immune (●) or maleyl-Ig-immune (○) mice to titrating doses of Ag either on day 8 (continuous lines) or day 70 (broken lines) postimmunization. *B*, Serum anti-CA Ab levels (mean ± SE) in Ig-immune or maleyl-Ig-immune mice immunized with CA-alum on day 70 after Ig or maleyl-Ig immunization, respectively. *C*, The anti-CA-proliferative responses from the CA-immunized mice in *B*, preimmunized with either Ig (●) or maleyl-Ig (○). *D*, Serum Ig levels in irradiated syngeneic recipients after adoptive cell transfer. Ig levels (mean ± SE) in recipient mice before transfer (−) and 7 days after transfer of normal B cells with T cells from the various groups are shown. Data are representative of three independent experiments.

any response to in vitro stimulation with Ig or maleyl-Ig (Fig. 6*A*). Although T and B cell numbers are normal in mice immunized with Ig or maleyl-Ig on day 70 postimmunization (data not shown), it is possible that they may not be functionally normal. We examined the ability of these mice to respond to an unrelated Ag, CA. Fourteen days after immunization with CA on alum, mice from Ig-immune and maleyl-Ig-immune groups showed comparable levels of CA-specific Ab responses (Fig. 6*B*) as well as in vitro T cell-proliferative response (Fig. 6*C*), suggesting that the loss of autoreactivity-associated phenomena was due to a specific functional loss of autoreactive T cells rather than to any global alteration in the functionality of the T or the B cell compartments.

To analyze the abilities of Ig-specific T cells to induce polyclonal activation of naive B cells in vivo and to study their fate, T cells purified from Ig-immune or maleyl-Ig-immune mice 14 or 70 days after immunization were adoptively transferred along with normal B cells into syngeneic γ-irradiated recipients. The serum Ig levels in the recipients were then monitored over time. Fig. 6*D* shows that although mice receiving T cells from day 14 maleyl-Ig-immune donors showed marked hypergammaglobulinemia ($p < 0.05$), mice receiving T cells from day 70 maleyl-Ig-immune donors or from both days 14 and 70 Ig-immune donors showed normal Ig levels. These data indicate that only T cells from day 14

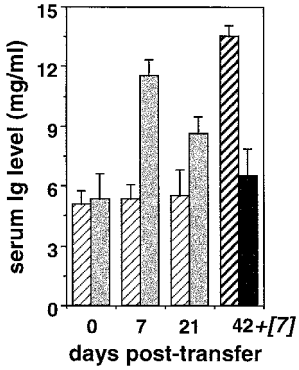

FIGURE 7. Ig-specific T cells are inactivated in vivo after maleyl-Ig immunization. Serum Ig levels (mean ± SE) are shown at various times in recipients after receiving normal B cells and T cells from either Ig-immune (▨) or maleyl-Ig-immune (shaded bars) mice 14 days after immunization. The last point in the series indicated by 42+ *[7]* and bold bars indicates serum Ig levels in the recipients 7 days after they were immunized with maleyl-Ig on day 42 posttransfer. Data represent three independent experiments.

maleyl-Ig-immune mice are capable of triggering naive B cells to produce hypergammaglobulinemia.

The serum hypergammaglobulinemia seen in mice receiving day 14 T cells from maleyl-Ig-immune donors declined over time (Fig. 7) and by day 42 serum Ig levels had returned to pretransfer levels (data not shown), whereas there was no change in serum Ig levels in the day 14 Ig-immune T cell recipients. Both of these groups were then immunized with 300 μg/mouse of maleyl-Ig and serum Ig levels were estimated 7 days later. Interestingly, recipients of maleyl-Ig-immune T cells did not show any change in serum Ig levels (Fig. 7), whereas recipients of Ig-immune T cells showed distinct hypergammaglobulinemia ($p < 0.02$), suggesting that once triggered by maleyl-Ig, Ig-specific T cells respond and cause polyclonal B cell stimulation, but at the same time become rapidly inactivated themselves.

Discussion

We have shown earlier that SR-mediated delivery of foreign protein Ags results in the enhancement of immunogenicity (36), and that the immune responses so generated show a relative Th1 dominance (35). The peptide repertoires generated from native protein and maleylated protein are largely cross-reactive (35). Those results suggested that higher densities of peptide-MHC complexes generated on all three major APC types, B cells, macrophages, and dendritic cells, and/or their persistence for longer periods in vivo may be relevant factors for regulating the type of immune response triggered. We had also shown previously that SR-mediated delivery of maleylated MSA can activate self-albumin-specific T cells (37), suggesting that such APC-targeted high-efficiency delivery of self-Ags may be instrumental in overcoming T cell tolerance. However, in spite of the presence of MSA-reactive T cells in vivo, no signs of autoimmune phenomena could be detected in these mice in vivo. One reason for this could be that the autoreactive T cells generated by maleylated self-protein immunization are poor-efficiency low-affinity leftovers from negative selection that can show some evidence of activation under ideal conditions in vitro but cannot function in vivo. An alternative possibility was that anti-self-albumin T cell responses do not cause autoimmune consequences in vivo because of the possibly restricted distribution of their peptide-MHC ligands, but that a similar break in T cell tolerance against some other self-Ags may do so. To test this possibility, we chose another ubiquitous self-protein, Ig, which, unlike

MSA, is synthesized and presented with high efficiency (48) by one class of professional APCs, B cells. Our results presented here show that maleyl-Ig-immune mice show not only a break in T cell tolerance detectable in vitro, but also show autoimmune consequences in vivo, although these autoimmune responses are short-lived.

As a self-Ag source, we have used Ig isolated from pooled syngeneic serum of BALB/c mice. However, it is quite possible that there would be idiotypic differences in Ig from individual mice, and therefore such pooled syngeneic Ig may be part "foreign" to any recipient of immunization. Therefore, in addition to using pooled Ig in both native and maleylated forms as recall Ags in vitro, we used preimmune autologous serum from each individual mouse as an additional source of Ig that would be completely self. Splenic cell proliferation assays done individually in this fashion unequivocally demonstrate break in tolerance to self-Ig (Fig. 1). We have previously shown that maleylated foreign proteins are not nonspecific mitogens for T cells, and the data shown in Fig. 1*C* confirm that this is true for maleyl-Ig as well.

The results from our previous experiments with triggering T cell autoreactivity to mouse albumin suggested that the presence of activated albumin-reactive T cells in these mice had no discernible pathophysiological consequences. The trivial explanation for this would have been that the autoreactive T cells triggered by maleylated self-protein immunization are low-affinity, low-efficiency cells that have no physiological significance. The fact that maleyl-Ig immunization leads to increased serum Ig levels in vivo (Fig. 1*D*), although immunization with maleyl-MSA does not, suggests that the trivial explanation is not tenable. It is much more likely that such autoreactive T cells are indeed capable of functioning in vivo, and that the identity of the self-protein concerned is decisive in determining the extent and outcome of their activity in vivo. The hypergammaglobulinemia is likely to be the result of polyclonal activation of B cells regardless of their antigenic specificity, since the levels of maleyl-Ig-specific Abs generated (<50 μg/ml; data not shown) are far lower than the increases observed in serum Ig levels. One potential implication of this finding is that Ig-derived peptide-MHC complexes are presented far more efficiently by B cells than MSA-derived peptide-MHC complexes are, despite the high concentrations of circulating serum albumin, raising issues of the relative efficiency of natural peptide generation from APC-endogenous vs APC-exogenous sources.

For SR-mediated uptake, Ags in solution form are delivered efficiently. We have used alum as an adjuvant in some of the present experiments (Fig. 1), since we have observed that adsorption of maleylated foreign proteins on alum still leads to increased uptake into and presentation by macrophages as well as to enhanced immunogenicity and a Th1 cytokine bias (data not shown). However, similar results have also been observed with immunization using maleyl-Ig in PBS alone (Fig. 2). We have also shown previously that, in addition to macrophages, some DCs and most B cells can bind to SR ligands such as maleylated proteins and present them to T cells (35). DCs are considered essential and sufficient APCs to prime for foreign Ags (49, 50). The APC requirements for the present break in tolerance are not clear. It was therefore of interest to ask whether macrophage depletion in vivo could alter the efficiency of this break in tolerance. Since CGN is known to deplete macrophages in vivo (42, 51), we immunized mice with maleyl-Ig during a period when splenic macrophages were eliminated by CGN treatment, leaving DC and B cell populations unaffected (data not shown). The results show (Fig. 2*A*) that macrophage depletion results in a decrease in the degree of autoreactivity triggered by maleyl-Ig (Fig. 2*A*) and maleyl-MSA (data not shown). The use of desulfated CGN, which induces less toxicity and affects macrophages more specifically than CGN (42, 51), gave similar results, both for maleyl-Ig and maleyl-MSA (data not shown). Despite macrophage depletion, carrageenan-treated mice immunized with maleyl-Ig do show some T cell-proliferative responses in vitro (Fig. 2*A*), possibly due to the fact that while DCs are not numerically or functionally affected by CGN treatment (Fig. 2, *B* and *C*), only a subpopulation of DCs shows the ability to bind maleylated proteins (35), possibly leading to poorer DC-mediated uptake and presentation of maleyl-Ig to T cells. These data suggest that functional macrophages contribute significantly to deliver and/or amplify the tolerance-breaking signals to autoreactive T cells, although it is not yet clear whether this is mediated by simply larger amounts of peptide-MHC complexes being generated or by alterations dependent on costimulatory signals specific to macrophages.

Using maleylated Ig to break T cell tolerance to Ig raises the possibility that new epitopes generated on the modified Ig molecule may in part be responsible for the triggering of autoreactivity, and that the SR-specific delivery may not be crucial. Therefore, we used an alternate modality of Ig delivery to SRs without maleylation of the Ig itself by coupling it to a carrier without any T cell epitopes, poly-L-lysine, that could be maleylated for SR-specific delivery. Maleyl-poly-L-lysine-coupled Ig did indeed induce hypergammaglobulinemia whereas poly-L-lysine-coupled Ig did not, and the need for specific delivery of Ig to SR-bearing APCs for inducing a break in tolerance is clearly shown by the competition in vivo of maleyl-poly-L-lysine-Ig with poly(G) (but not poly(C)) leading to reduced hypergammaglobulinemia (Fig. 2*D*).

Since mice showing a break in T cell tolerance for autologous Ig show significant increases in their serum Ig levels, it appears that once triggered, Ig-specific T cells can recognize Ig-derived peptide-MHC complexes on B cells and activate them. Because it is quite possible that the self-Ig peptides being recognized by these autoreactive T cells are invariant, a single Ig-specific T cell may be able to activate many B cells presenting the same Ig-derived peptide, resulting in B cell differentiation causing hypergammaglobulinemia. This activation appears to extend to isotype switching, since both IgM and IgG levels in serum are elevated (Fig. 3). The extent of hypergammaglobulinemia is comparable between various IgG subclasses (data not shown), emphasizing the diversity of B cells being activated. Also, the weight and the cellularity of the spleen increase in maleyl-Ig-immune mice (Fig. 3), further reinforcing the extensive nature of the alterations taking place in vivo.

The polyclonality of the B cells being activated is also evident in the phenotypic characterization of splenic B cells (Fig. 4). Within a few days of maleyl-Ig immunization, most splenic B cells down-modulate their surface IgD and acquire increased PNA-binding capabilities, showing that they are being activated by T cells. In addition, many of these B cells begin to coexpress the plasma cell marker CD138, indicating that they may be in transition to plasma cell differentiation. In fact, by day 14 postimmunization, this process appears to be well advanced, since the proportion of B cells in the spleen dwindles and $B220^-$ plasma cells become a prominent population (Fig. 4). Thus, maleyl-Ig-triggered Ig-specific T cells appear to be responsible for T-dependent activation of most B cells, leading to hypergammaglobulinemia within 2 wk after immunization.

However, despite the continuous presence of endogenous Ag available for triggering Ig-specific T cells, the hypergammaglobulinemia is transient (Fig. 5*A*) as are the splenic hypercellularity (Fig. 5*B*) and the activated phenotype of splenic B cells (Fig. 5*C*). There are no further pathological consequences such as renal damage that we have been able to observe in these mice either (data not

shown), possibly owing to the transient nature of the hypergammaglobulinemia. In fact, the rate of decline of the serum Ig levels is rapid enough that, considered together with the known half-life of circulating Ig, it is possible that all B cells responding in a small time window go through terminal differentiation to plasma cells. This transience appears to be a consequence of the rapid loss of functional Ig-specific autoreactive T cells, so that by 10 wk postimmunization, there are no T cells in the mice that can either proliferate in response to self-Ag or provide help to B cells (Fig. 6). Thus, once the initial B cells activated by triggered Ig-specific T cells have gone to terminal plasma cell differentiation, the new B cell populations emerging from the bone marrow are no longer stimulated by autoreactive T cells. These B cells, which show a normal phenotype at day 70 postimmunization, are functionally normal, since they respond normally to further immunization with a T-dependent protein Ag (Fig. 6*B*).

The adoptive transfer experiments demonstrate that the Ig-specific autoreactive T cells induced by maleyl-Ig immunization can drive normal B cells to Ig secretion without the continuing presence of maleyl-Ig (Fig. 6*D*). These experiments are also supported by data on adoptive transfer of T cells from variously immunized donors into nude mice (data not shown). However, this property is lost by 10 wk postimmunization, so that there seem to be no autoreactive Ig-specific T cells left by this time point (Fig. 6*D*). In fact, reimmunization of the recipients shows that, although a transferred naive T cell repertoire (from the Ig-immunized mice) is perfectly capable of responding against a first exposure to maleyl-Ig, previous experience with maleyl-Ig renders the T cells taken from maleyl-Ig-immune mice unable to mount an autoreactive response again (Fig. 7). Clearly, the induction of anti-Ig T cell autoreactivity is followed by rapid inactivation of the Ig-specific T cells making the mice tolerant to further autoimmunization. Peripheral induction of T cell tolerance following activation has been documented in many instances such as with superantigens (52, 53) where T cell activation is followed by clonal exhaustion and apoptotic cell death. It is quite plausible that presentation of Ig-derived peptides in large amounts by activated B cell APCs might result in hyperstimulation of Ig-specific T cells leading to similar functional inactivation. However, it is also possible that autoreactive T cells are being subjected to some regulatory mechanism such as "infectious tolerance" (54), since the maleyl-Ig-specific T cell response triggered in the recipients from the reconstituting endogenous T cells appears to be poor even 42 days after transfer (Fig. 7).

These data clearly show that immunization with a maleylated self-protein can bring about a break in T cell self-tolerance with autoimmune consequences in vivo. Whether the Ag is endogenous to professional APCs or not may be an important factor for determining the pathologic effects of such a break in tolerance. However, the very fact that endogenous Ag is presented in high enough concentrations to induce detectable effects in vivo may lead to clonal exhaustion of the autoreactive T cells, limiting the duration of the consequences induced. It is attractive to speculate that this may be a factor contributing to the relapse and remission progression of many autoimmune diseases.

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